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Manufacture of a beverage from cheese whey using a "tea fungus" Fermentation

Genette Belloso-Morales,* Humberto Hernández-Sánchez*

ABSTRACT. Kombucha is a sour beverage reported to have potential health effects prepared from the fermentation of black tea and sugar with a "tea fungus", a symbiotic culture of acetic acid bacteria and yeasts. Although black tea is the preferred substrate for Kombucha fermentation, other beverages have also been tested as substrates with fair results. Cheese whey is a by-product with a good amount of fermentable lactose that has been used before in the production of beverages, so the objective of this study was to test three types of whey (fresh sweet, fresh acid and reconstituted sweet) in the elaboration of a fermented beverage using a kombucha culture as inoculum. The isolation and identification of bacteria and yeasts from the fermented tea and wheys was done along with the study of the rates of change in sugar consumption, acid production and pH decrease. Several species of acetic acid bacteria (*Acetobacter aceti* subsp. *aceti*, *Gluconobacter oxydans* subsp. *industrius*, subsp. *oxydans* and *Gluconoacetobacter xylinus*) were isolated from the different kombuchas along with the yeasts *Saccharomyces cerevisiae*, *Kluyveromyces marxianus*, and *Brettanomyces bruxelensis*. The main metabolic products in the fermented wheys included ethanol, lactic and acetic acids. A good growth was obtained in both sweet wheys in which a pH of 3.3 and a total acid content (mainly lactic and acetic acids) of 0.07 mol/l was reached after 96 h. The sweet whey fermented beverages contained a relatively low lactose concentration (< 12 g/l). The final ethanol content was low (5 g/l) in all the fermented wheys. The whey products were strongly sour and salty non sparkling beverages.

Key words: Whey beverages, kombucha, "tea fungus", fermentation.

RESUMEN. La kombucha es una bebida agria, con posibles efectos benéficos para la salud, preparada por fermentación de té negro azucarado con el llamado "hongo del té", un cultivo simbiótico de bacterias acéticas y levaduras. Aunque el té negro es el mejor sustrato para la fermentación de la kombucha, también se han probado otras bebidas como substratos con buenos resultados. El suero de queso es un subproducto con una buena cantidad de lactosa fermentable que se ha usado en la producción de bebidas, por lo que el objetivo de este estudio fue el de probar tres tipos de lactosuero (dulce fresco, ácido fresco y dulce reconstituido) en la elaboración de bebidas fermentadas usando un cultivo de kombucha como inóculo. Se realizó el aislamiento e identificación de las bacterias y levaduras de los sueros fermentados junto con el estudio cinético de consumo de azúcares, producción de ácido y disminución de pH. Se aislaron varias especies de bacterias acéticas (*Acetobacter aceti* subsp. *aceti*, *Gluconobacter oxydans* subsp. *industrius*, subsp. *oxydans* y *Gluconoacetobacter xylinus*) de las diferentes kombuchas junto con las levaduras *Saccharomyces cerevisiae*, *Kluyveromyces marxianus* y *Brettanomyces bruxelensis*. Los principales productos metabólicos presentes en los sueros fermentados fueron etanol y los ácidos láctico y acético. Se obtuvo un buen crecimiento en los dos sueros dulces, alcanzándose un pH de 3.3 y una acidez total (constituida por los ácidos acético y láctico) de 0.07 mol/l después de 96 h. Las bebidas de los sueros dulces tuvieron un contenido relativamente bajo de lactosa (< 12 g/l). El contenido final de etanol fue bajo (5 g/l) en todos los sueros fermentados, teniendo un fuerte sabor agrio y salado y careciendo de efervescencia.

Palabras clave: Bebidas de lactosuero, kombucha, "hongo del té", fermentación.

INTRODUCTION

Around 135 million kg of cheese are produced annually in Mexico, resulting in over 675 million kg of whey. Though a small amount may be used in the food industry or as a feed, the whey, in general, is thrown into streams and the sewer systems or disposed of in other ways creating environmental and economic problems. However, for many years whey has provided the base for a wide variety of beverages. To some people, whey is a waste, but to others it represents an opportunity for new food development.[9] A new approach to transform whey into a palatable beverage could be the "tea fungus" fermentation.

The "tea fungus" is an association of yeasts and acetic acid bacteria which can ferment a mixture of black tea and sucrose into a sour, slightly sparkling refreshing beverage known as kombucha, haipao or teakwass.[2,10,12,14] The product is consumed world-wide and has become very popular due to controversial health claims. It is said that its consumption can lower blood pressure, relieve arthritis, psoriasis, chronic fatigue, constipation, indigestion and metabolic diseases, increase the immune response, and cure cancer.[6,16] The preparation of kombucha follows different recipes, but it is commonly made by infusing black tea leaves into hot water, sweetened with 5 to 15% sucrose, for around 10 min. After cooling to room temperature it is inoculated with the "tea fungus" and allowed to ferment for about 7 to 10 days.[3,6] The symbiotic culture of acetic acid bacteria and yeasts produces a zoogleal cellulose mat that resembles a surface mold and which helps in the aeration process of the fermentation due to an increased oxygen transfer surface.[3]

* Departamento de Graduados e Investigación en Alimentos, Escuela Nacional de Ciencias Biológicas, Instituto Politécnico Nacional, Carpio y Plan de Ayala, 11340 México, D. F., México.

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The microorganism composition of the "tea fungus" is highly variable and depends strongly on the source of the culture. The most commonly reported acetic acid bacteria in the culture are: *Acetobacter aceti*, *A. pasteurianus*, *"A. xylinoides"* and *Gluconoacetobacter xylinus*.[2,3,6,10,12-14] The yeast spectrum of the "tea fungus" is even more variable: *Brettanomyces bruxellensis*, *B. intermedius*, *Candida famata*, *Pichia membranofaciens*, *Saccharomyces cerevisiae*, *Saccharomycodes ludwigii*, *Schizosaccharomyces pombe*, *Torulaspora delbrueckii*, *Zygosaccharomyces bailii*, and *Z. Rouxii*.[2,3,6,7,10,12,14] Although black tea is the preferred substrate for kombucha fermentation, other beverages have also been tested as substrates with fair results.[6] There are studies on the influence of different sugars on the metabolism of the "tea fungus". When lactose was used as a carbon source, the final products of fermentation were acetic and lactic acids as well as ethanol.[12] Since lactose can be used as substrate by the "tea fungus", the objective of this study was to explore the possibility of using three kinds of whey for making a fermented beverage using a Kombucha culture as inoculum.

MATERIALS AND METHODS

Biological and raw material. Fresh sweet and acid wheys were obtained in the laboratory from the experimental manufacture of Panela and Guayanés cheeses, respectively.[5] Sweet whey powder was purchased from Davisco Foods International, Inc. through a dealer in Mexico City and was reconstituted to 7% solids with distilled water. In all cases and before fermentation, the wheys were heated to 117°C for 15 min, allowed to cool to room temperature, and filtered through Whatman 1 paper to obtain supernatant fluids.[4] A black tea infusion was also prepared to be able to compare the behavior of the fungus in the wheys and in its preferred substrate. Eight g of black tea (Lipton bags) were placed in sterile hot (90°C) water and allowed to cool for 45 min. Sucrose was added to a final concentration of 70 g/l. The "tea fungus" was bought in a health food store in Mexico City.

Culture conditions. Erlenmeyer flasks with 250 ml of the wheys or black tea were inoculated with 20 g of "tea fungus", coming from a mat cultured in black tea, and incubated at 32°C without agitation for 4 days (all the experiments were done in triplicate).

Isolation and identification of microorganisms. One g of the mat was taken with a sterile spatula for the isolation of the bacteria and yeasts from the wheys and tea cultures. The samples were dispersed in saline solution, serially diluted and spread on the appropriate media, which were plate count agar, trypticase soy agar and MRS agar (polypeptone 10 g, meat extract 10 g, yeast extract 5 g, glucose 50 g, Tween 80, 1 ml, K_2HPO_4 2 g, sodium acetate 5 g, triammonium citrate 2 g, $MgSO_4$. $7H_2O$ 0.2 g, $MnSO_4.4H_2O$ 0.05 g, agar 20 g and water 1 L) for the bacteria (the addition of antimycotics was not necessary) and corn meal agar and malt extract agar for the yeasts and incubated under aerobic conditions at 28°C for 48 - 72 h in the case of yeasts and at 32°C for 48 h in the case of bacteria. All the culture media were purchased from Becton Dickinson Bioxon™ (Cuautitlán, México). The microorganisms were identified using morphological (colonial and cell morphology and Gram's stain) and physiological methods (fermentation of carbohydrates and related compounds). The differentiation of acetic acid bacteria was initially made with the assays for overoxidation of ethanol to acetic acid (positive for *Acetobacter* and *Gluconoacetobacter* and negative for *Gluconobacter*) and oxidation of DL-lactate to CO_2 and H_2O and of acetate to CO_2 and H_2O (also positive for *Acetobacter* and *Gluconoacetobacter* and negative for *Gluconobacter*). In the case of bacteria, the growth on starch, ethanol, arabinose, cellobiose, inulin, maltose, raffinose, sorbitol, sorbose, xylose, glucose, lactose, mannitol and sucrose was tested. In the case of yeasts, the fermentation of glucose, cellobiose, lactose, maltose, raffinose, and sucrose and the aerobic growth assimilation of arabinose, erythritol, glucosamine, inulin, melezitose, rhamnose, sorbitol, sorbose, trehalose, starch, and mannitol were tested. The results were analyzed according to the procedures described on Bergey's Manual[8] and by Barnett *et al.*[1]

Analysis of substrates and metabolites. Total plate count after 96 h of fermentation was determined in Standard Methods Agar. Whey and supernatant fluids were analyzed for protein by use of the Kjeldahl procedure.[4] The pH was measured with a potentiometer (Orion 920, Boston, MA). Total acidity was determined by titration with NaOH 0.1N and expressed in moles/l.[19] Lactose, glucose and galactose were determined by the colorimetric method of Nickerson *et al.*[11] only in the cases in which whey was the substrate. Ethanol was determined in the fermented beverages (after deproteinization with 6.25% trichloroacetic acid solution) using alcohol dehydrogenase and nicotinamide adenine dinucleotide. Absorbance at 340 nm, which occurs when NAD is converted to NADH, is an accurate measure of the amount of ethanol present.[15] Acetic and lactic acid were evaluated by HPLC under the following conditions given by the column supplier and by Blanc:[3] Rezex 8u 8% H column for organic acids (Phenomenex, Torrance, CA), 0.005N H_2SO_4 as the mobile phase, a flow rate of 0.4 ml/min, room temperature and a UV-Vis detector working at 210 nm . All the analyses were done in triplicate and the average of the determinations is shown in the results. When differences between lots or samples were to be shown, one-way ANOVA was performed.

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RESULTS

Isolation and identification of microorganisms

Four different isolates (IT1, IT2, IT3 and IT4) of bacterial colonies were obtained from the 96 colonies picked randomly from the several plates obtained from the "tea fungus" colony grown in black tea. All of them showed the morphological characteristics of the acetic acid bacteria, that is, the cells stained Gram negative, and were ellipsoidal to rod-shaped, 0.5 to 1.0 μm X 1.0 to 4.0 μm, occurring singly, in pairs, and sometimes in chains.[8] All the isolates were able to oxidize ethanol to acetic acid in neutral or acidic (pH 4.5) media. There are only five aerobic Gram-negative bacteria that can oxidize ethanol to acetic acid in neutral or acidic media and they include *Acetobacter, Acidomonas, Frateuria, Gluconoacetobacter* and *Gluconobacter*. Table 1 shows the results of the preliminary tests for the isolates and the type strains which were determinant in the elucidation of the genus of each isolate. It can be concluded that the only microorganisms which could be present in the isolates belong to the genera *Acetobacter, Gluconoacetobacter* and *Gluconobacter* (IT1, IT2 and IT4). After the physiological methods were performed, the following results were obtained: the first isolate (IT1) showed 80% correspondence in the biochemical tests with *Gluconobacter oxydans* type strain ATCC 19357 (i.e. 80% of the 14 biochemical tests were identical to the results for the type strain), the second (IT2) 90% with *G. oxydans*, the third (IT3) 85% with *Acetobacter aceti* type strain ATCC 15973 and the fourth (IT4) 80% also with *G. oxydans*. Two different strains (IW1 and IW2) of *Gluconoacetobacter* were isolated from the 32 colonies picked at random from the various plates obtained from the "tea fungus" culture grown in the wheys (see Table 1). *Gluconoacetobacter* can be differentiated from *Acetobacter* in its inability to grow on ethanol or D-mannitol as carbon sources. The first one (IW1) showed 100% correspondence with *Gluconoacetobacter xylinus* type strain ATCC 23767 (which is lac-) and the second (which is lac^+) 70% also with this microorganism. *Gluconoacetobacter xylinus* (formerly known as *Acetobacter xylinum* and the only recognized species of the genus) is a cellulose-forming acetic acid bacteria[18] which then could be responsible for the formation of the structure of the fungus mat. *Gluconoacetobacter* was elevated to the generic level based on partial sequences of 16S ribosomal RNA.[20]

Three different isolates of yeasts were obtained from the 92 yeast colonies picked at random from the isolates from the "tea fungus" colony grown in black tea. After the morphological and physiological tests were performed, 37.5% of the tea yeast population belonged to *Saccharomyces cerevisiae* type strain ATCC 18824, 25% to *Brettanomyces bruxelensis* (which is the asexual state of *Dekkera bruxelensis*) type strain NRRL Y-1411 and 37.5% to *Kluyveromyces marxianus* type strain NRRL Y-8281. In the case of the yeasts isolated from the "tea fungus" grown in the different kinds of whey, the microorganisms were basically the same, although the proportions varied. In the reconstituted sweet whey, 60% of the population belonged to *Brettanomyces bruxelensis*, a yeast which can ferment the lactose, 20% to *Kluyveromyces marxianus* (also a lactose-fermenting yeast) and 20% to *Saccharomyces cerevisiae* (which is lac-). In the fresh sweet whey, 75% of the population was *Saccharomyces cerevisiae* and 25% *Kluyveromyces marxianus*, and in the acid whey, 87.5% was *Saccharomyces cerevisiae* and 12.5% *Kluyveromyces marxianus*.

A total yeast count of 1.34 X 10^7 CFU/ml and a total bacterial count of 6 X 10^5 CFU/ml were obtained in the case of the black tea Kombucha after the four days fermentation period. The ratio of yeasts to acetic acid bacteria was 23:1. When the reconstituted sweet whey beverage was analyzed, a total count of 9 X 10^6 CFU/ml was obtained with a ratio of yeasts to bacteria of 5:1. The same ratio was obtained in the sweet and acid whey products with total counts of 9 X 10^6 and 8 X 10^6 CFU/ml respectively after four days of fermentation.

Fermentation studies

After 96 h of fermentation, the following total plate counts were registered in the fermentation media: 1.4 X 10^7 CFU/ml for the black tea, 9 X 10^6 CFU/ml for the reconstituted sweet

Table 1. Differentiation of the aerobic Gram-negative bacterial isolates that oxidize ethanol to acetic acid in neutral or acidic (pH 4.5) media.

Characteristic	*Acetobacter*	*Acidomonas*	*Frateuria*	*Gluconoacetobacter*	*Gluconobacter*	IT1	IT2	IT3	IT4	IW1	IW2
Overoxidation of ethanol	+	?	–	+	–	–	–	+	–	+	+
Oxidation of lactate to CO_2 and H_2O	+	–	+	+	–	–	–	+	–	+	+
Oxidation of acetate to CO_2 and H_2O	+	+	–	+	–	–	–	+	–	+	+
Acid from arabinose	–	?	+	–	+[a]	–	–	–	–	–	–
Acid from maltose	–	–	–	–	+ or –	+	+	+	+	–	+

[a] Some strains are negative

IT1, IT2, IT3 and IT4 are isolates from the tea and IW1 and IW2 are isolates from the whey

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whey, 9 X 10^6 CFU/ml for the fresh sweet whey and 8 X 10^6 CFU/ml for the fresh acid whey. The main physical difference in the colonies was in the texture. The mat formed in the black tea was the typical soft fungus-like structure, whereas the mats obtained in the three wheys had a cardboard-like coarser structure.

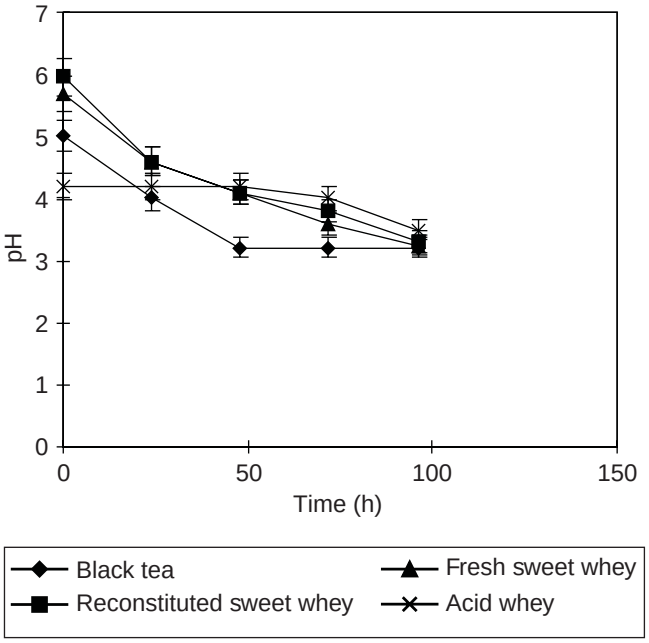

Figure 1. *Rate of change in pH during the "tea fungus" fermentation of black tea and three kinds of whey.*

Fig. 1 shows the decrease in pH with time for the four substrates. The kinetics of increase in total titratable acidity (expressed as acetic acid for the black tea and as lactic acid for the wheys) is shown in Fig. 2. The ethanol content in the four substrates after the 96 h fermentation was 5 g/l. Fig. 3 and 4 show the kinetics of lactose and glucose + galactose degradation respectively in the whey beverages.

The variation of lactic and acetic acids during the fermentation is shown in Fig. 5 and 6 respectively for the four substrates.

DISCUSSION

Isolation and identification of microorganisms

The results for the acetic acid bacteria isolated from the black tea Kombucha were similar to those of Liu *et al.*, since no *Gluconoacetobacter xylinus* could be isolated.[10] They also isolated *A. aceti*. However, in the case of the whey, two strains of *Gluconoacetobacter* could be isolated. Thus, there was a change in the predominant kind of bacteria present in the whey. Since lactose is the main carbon source, a *Gluconoacetobacter* strain which can metabolize this sugar is present in higher proportion in the whey than in the tea.

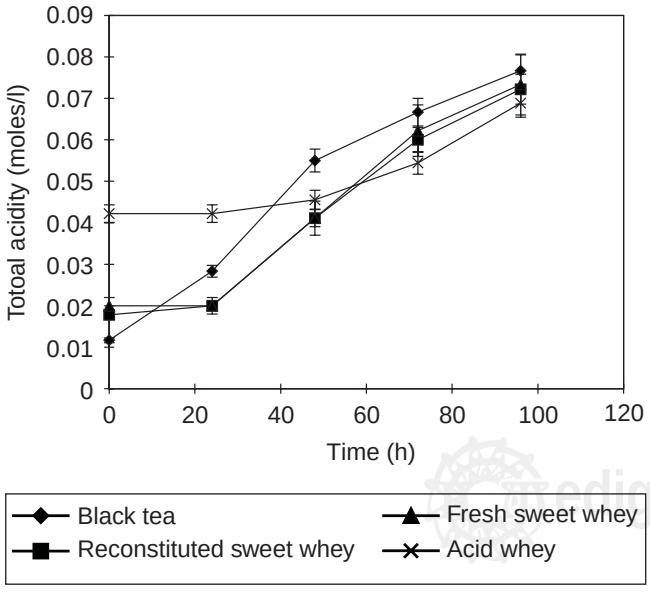

Figure 2. *Rate of change in total titratable acidity during the "tea fungus" fermentation of black tea and three kinds of whey.*

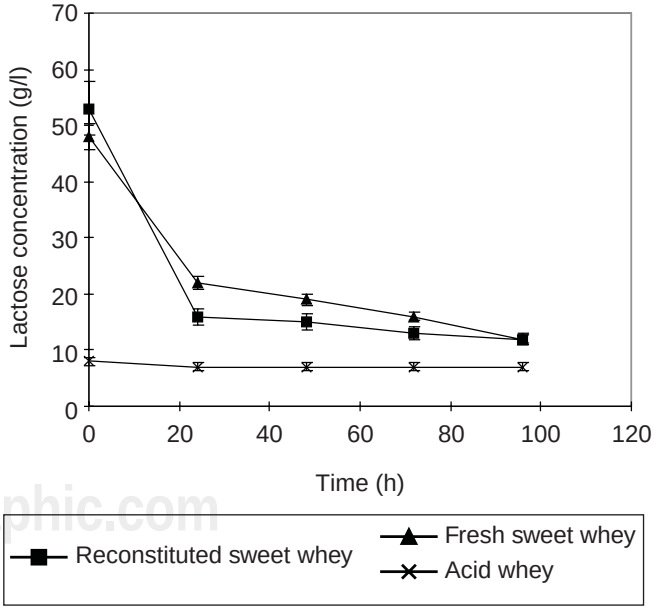

Figure 3. *Rate of decrease in lactose concentration during the "tea fungus" fermentation of three kinds of whey.*

Two of the yeasts (*Saccharomyces cerevisiae* and *Brettanomyces bruxelensis*) isolated from the black tea Kombucha were also reported to be present in the "tea fungus" by other authors.[7,10] In the case of the whey beverages, a better fermentation was to be expected in the reconstituted sweet whey, since 80% of the yeast population (*Brettanomyces bruxelensis* and *Kluyveromyces marxianus*) can ferment the lactose present in this cheese by-product.

From the total counts of bacteria and yeasts in the different products after four days of fermentation, it is evident that the change from tea to whey induced a decrease in the proportion of yeasts to bacteria. The higher total counts in the tea Kombuchas confirm the reports of other authors that the sugared black tea is the best substrate ($p < 0.05$) for the "tea fungus".[6] However, the "tea fungus" culture was able to grow well and similarly in the three kinds of whey since no significant difference could be found by ANOVA among the counts ($p > 0.05$) in these dairy by-products. The change in the texture of the colony in the wheys with respect to the colony in the tea may be a consequence of the change in the microflora, the change in metabolism or both as a result of the change in substrate.

Fermentation studies

In the case of pH decrease, the behavior of the black tea Kombucha is identical to the one reported by Blanc.[3] The pH drops more slowly in the wheys possibly due to the buffering effect of the residual proteins. The reconstituted sweet, acid and sweet wheys have protein concentrations of 0.89, 1.67 and 0.94% respectively, while the supernatant fluids have concentrations of 0.69, 1.11 and 0.67% respec-

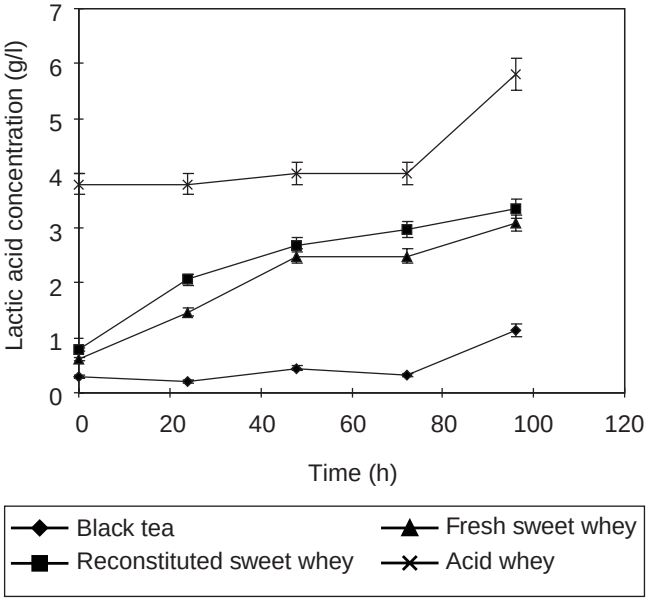

Figure 5. *Rate of lactic acid production during the "tea fungus" fermentation of black tea and three kinds of whey.*

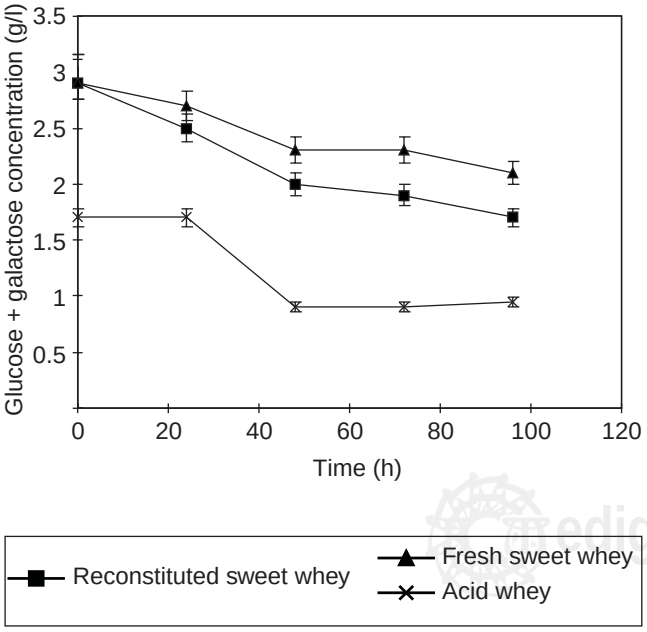

Figure 4. *Rate of change in glucose and galactose concentration during the "tea fungus" fermentation of three kinds of whey.*

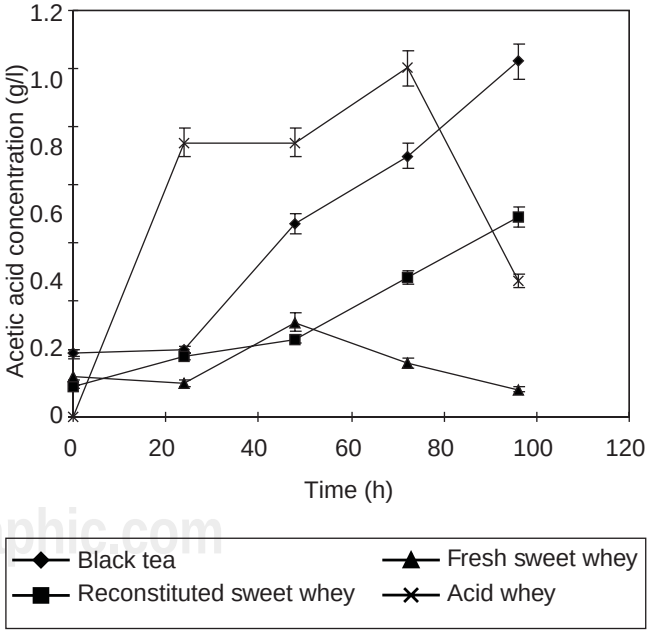

Figure 6. *Rate of acetic acid production during the "tea fungus" fermentation of black tea and three kinds of whey.*

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tively, so this hypothesis could be valid. The pattern of pH drop is very similar in the case of both sweet wheys; however, the acid whey has a different rate of change due to its lower initial pH (4.2) and sugar concentration (12 g/l).

In the case of acid production, again the behavior of black tea is very similar to that reported by Blanc.[3] The fresh sweet and the acid wheys reached about the same acidity after the 96 h of fermentation (6 g/l). The reconstituted sweet whey had the highest total acidity (15 g/l) at the end of the 96 h fermentation. The ethanol content is in the range (1.34 - 8.11 g/l) reported by other authors in black tea Kombucha.[3,12,14] Other authors did not detect ethanol in the Kombucha fermentation.[16]

In the fermentation studies that involved sweet whey, the rates of lactose degradation were very similar (Fig. 3), being very fast at the beginning and becoming slower afterwards. This pattern was also present, in the case of sucrose, in the black tea Kombucha fermentation.[3] The sweet whey fermented beverages contained a relatively low lactose concentration (< 12 g/l), making them acceptable for consumption by people with lactose intolerance. Acid whey showed only a small decline in lactose concentration since the initial value was very low. The same applies to the rates of consumption of glucose and galactose (Fig. 4), although in this case the degradation was not as fast as in the case of lactose and the initial and final values are relatively low compared with those of lactose.

The production of lactic acid (Fig. 5) in the black tea reached a maximum (1.15 g/l) after 96 h. The value is slightly higher than the one reported by other authors (0.6 - 0.9 g/l) under similar conditions.[3,12] In the case of the sweet wheys, the lactic acid concentration rose steadily to a maximum (3.1 - 3.4 g/l) after the 96 h of fermentation. The acid whey did not show an increase in this acid during the first 72 h. After this period, a sharp increase in the concentration was observed (5.8 g/l).

The acetic acid which was converted from ethanol increased steadily in the black tea reaching a maximum (1.05 g/l) after 96 h of fermentation. This value is lower than those reported by other authors (5 g/l after a 5-day fermentation by Blanc and 2.1 g/l after a 10-day fermentation by Sievers *et al.*) possibly indicating that the acetic bacteria present in this fungus tea cannot metabolize ethanol as efficiently as others.[3,14] In the case of the reconstituted sweet whey, the behavior was very similar to that of the black tea; however the maximum concentration reached only 0.6 g/l. In the other wheys, the acetic acid rose also to a maximum (0.28 g/l in the fresh sweet whey after 48 h and 1 g/l in the acid whey after 96 h) and subsequently declined (Fig. 6). This last behavior was observed by Blanc in the black tea Kombucha at different sucrose concentrations.[3] The differences between the total titratable acidity and the concentrations of lactic and acetic acids in the different substrates can be attributed to the presence of other acid metabolites like gluconic acid.[3,7,14] Unlike the black tea kombucha, the fermented wheys were not sparkling beverages, indicating that in these substrates, the production of carbon dioxide was minimal.

It can be concluded that the "tea fungus" can use the cheese whey as a substrate and that the best results were obtained with the reconstituted sweet whey. The fermented product was tasted by the authors and described as strongly sour and salty. It could be improved sensorially if the whey is previously demineralized and a fruit flavor is added at the end of the process

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Correspondence to:

Humberto Hernández-Sánchez
Departamento de Graduados e Investigación en Alimentos,
Escuela Nacional de Ciencias Biológicas,
Instituto Politécnico Nacional.
Carpio y Plan de Ayala, 11340
México, D. F., México
Tel. (52) 5729-6000 ext. 62458;
Fax. (52) 5729-6000 ext. 62359;
E-mail: hhernan@encb.ipn.mx